

Basic Science Reports

Adenovirus-Mediated Transfer of a Gene Encoding Human Apolipoprotein A-I Into Normal Mice Increases Circulating High-Density Lipoprotein Cholesterol

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Background In animal models of atherosclerosis, augmentation of circulating high-density lipoprotein (HDL) cholesterol exerts a protective effect against development of fatty streaks and promotes plaque regression.

Methods and Results To investigate the potential of gene transfer to increase HDL cholesterol, a fusion gene encoding human apolipoprotein A-I (apo A-I) under the control of the human cytomegalovirus (CMV) immediate-early promoter was packaged into a recombinant adenovirus (AdCMV apo A-I). BALB/c mice infected with AdCMV apo A-I by intravenous injection accumulate immunoreactive apo A-I in serum; levels 5 days after infection averaged 168 mg/dL. A 35% increase in HDL cholesterol and a 47% increase in total cholesterol were observed in mice infected with AdCMV apo

A-I compared with control viruses. Analysis of size-fractionated lipoproteins revealed that human apo A-I is incorporated into murine HDL particles. Expression of human apo A-I declined to <10% of maximum after 12 days and mRNA encoding apo A-I, prevalent 5 days after infection, was undetectable in the livers of infected mice after 12 days.

Conclusions We conclude that adenovirus-mediated transfer of a gene encoding apo A-I produces transient elevations of circulating HDL cholesterol of a magnitude correlated with important physiological effects. These observations suggest the potential for gene-based therapeutic strategies to reduce cardiovascular risk. (*Circulation*. 1994;90:1319-1327.)

Key Words • atherosclerosis • genes • viruses • lipoproteins • apolipoproteins

Epidemiological data demonstrate an inverse relation between circulating levels of high-density lipoprotein (HDL) cholesterol and the incidence of clinically significant atherosclerosis.¹⁻⁴ This relation holds for even small increments of HDL cholesterol, such that each 1-mg/dL increase in HDL cholesterol level is associated with a 2% to 3% decrement in cardiovascular risk.⁴ Experimental evidence also supports a protective effect of HDL against atherosclerosis. Cholesterol-fed rabbits treated by infusion of purified homologous HDL are protected against the development of fatty plaques despite unchanged circulating HDL cholesterol levels.⁵⁻⁷ This association between HDL cholesterol and the incidence of atherosclerotic vascular disease suggests that strategies to increase circulating HDL could have important clinical application. A modest increase in HDL cholesterol has been observed in patients treated with gemfibrozil,² an intervention associated with a reduced incidence of cardiac events. Trials intended to specifically assess the effects

of intervention to increase HDL cholesterol on the development and progression of atherosclerosis are in progress.^{8,9}

HDL appears to exert its antiatherogenic effect by mediating reverse cholesterol transport, in which cholesterol is mobilized from peripheral tissues and transported to the liver.¹⁰⁻¹² The small, high-density, pre- β subspecies of HDL, comprised predominantly of apolipoprotein (apo) A-I and phospholipid, is thought to act as the physiological acceptor for cholesterol in the extracellular matrix of peripheral tissues.¹¹ Peripheral availability of this "scavenger" particle appears to be regulated by the rates of synthesis, secretion, and catabolism of HDL.¹⁰⁻¹²

Both clinical and experimental data suggest that the principal protein constituent of HDL, apo A-I, mediates the antiatherogenic activity of HDL¹ and that the rate of production of apo A-I is a critical determinant of circulating HDL cholesterol. Families with both heritably deficient¹³⁻¹⁶ and enhanced¹⁷ apo A-I levels have been identified and show corresponding alterations in HDL cholesterol. Persons with familial hyperalphalipoproteinemia appear protected from atherosclerosis, while those deficient in apo A-I show accelerated cardiovascular disease. Mice transgenic for a copy of the human apo A-I gene demonstrate accumulation of human apo A-I in serum, increased circulating HDL cholesterol, and resistance to the atherogenic effects of a high cholesterol diet.¹⁸⁻²¹ Thus, while the mechanisms regulating the rate of apo A-I synthesis are not clearly defined, genetic factors appear to exert an important effect.²²

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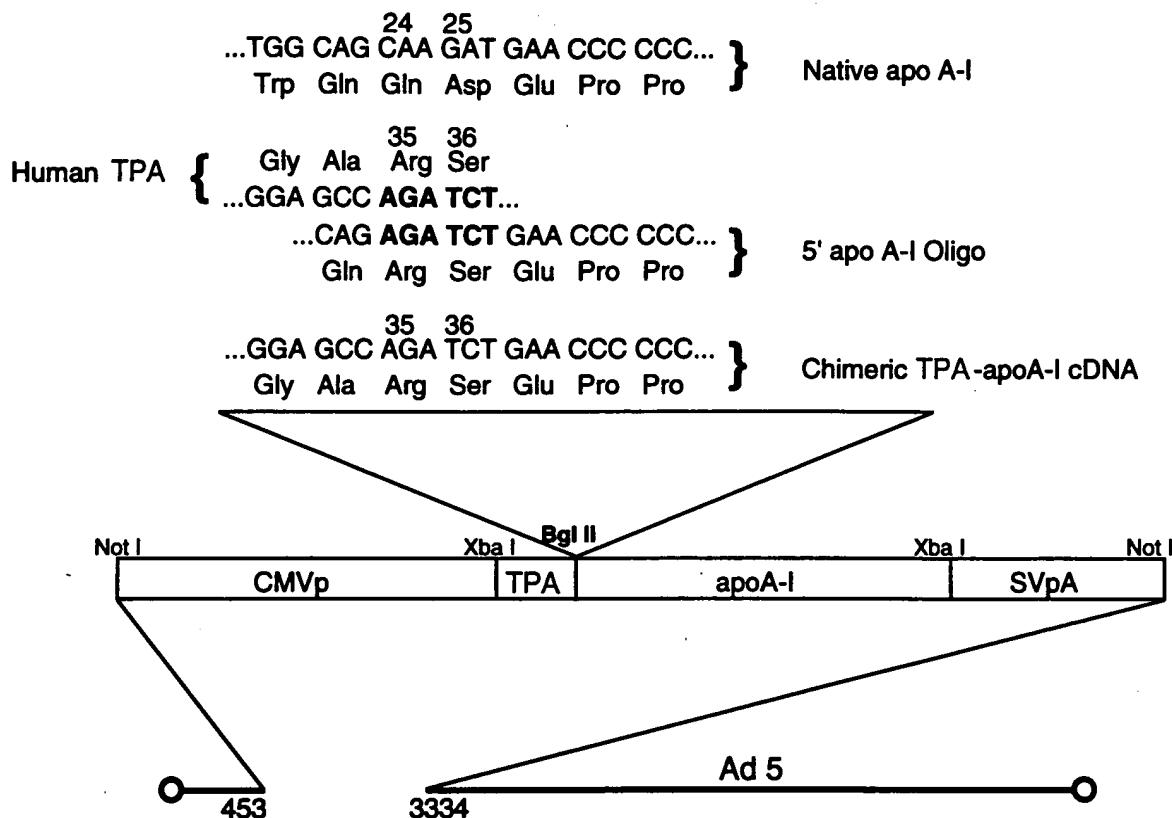


FIG 1. Chart of the construction of AdCMV apolipoprotein (apo) A-I. A fragment of the cDNA encoding human tissue plasminogen activator (TPA) and a partial cDNA encoding the mature human apo A-I protein were ligated in frame using an artificial BgIII site (shown in bold type) introduced into the apo A-I sequence during amplification. The resulting chimeric cDNA encodes the mature form of apo A-I behind the secretory signal sequence of human TPA (residue numbers as shown). This cDNA was inserted into pACCMVpLPAp between the human cytomegalovirus immediate-early promoter and transcriptional termination and polyadenylation sequences derived from SV40 to form a complete transcriptional unit. The recombinant adenovirus AdCMV apo A-I was generated by homologous recombination between pACCMV apo A-I and pJM17 after transfection into 293 cells.

In view of these observations, somatic cell gene transfer to augment apo A-I expression offers an attractive investigational and potentially therapeutic approach. In this article, we report that normal mice infected with a recombinant adenovirus encoding human apo A-I express high levels of human apo A-I in serum. These animals demonstrate increases in circulating HDL cholesterol similar to those observed in mice transgenic for a copy of the human apo A-I gene and of a magnitude previously associated with a protective effect against the development and/or progression of experimental atherosclerosis.

Methods

Generation of Recombinant Adenoviruses

The recombinant adenovirus (cytomegalovirus, CMV) Ad-CMV apo A-I was generated by the strategy illustrated in Fig 1. A cDNA encoding mature human apo A-I was obtained by polymerase chain reaction from a human liver cDNA library (generously provided by David Russell) using the oligonucleotide primers 5':CGGCATTTCTGGCAGAGATCTGAAC-CCCCCCAGA:3' and 5':TTTTCTAGAGCCCTCACTGGGT-GTTGAGACTTCTT:3'. The underlined sequences correspond to positions (relative to the translational start) +55 to +89 and +764 to +788 in the previously reported human apo A-I cDNA.^{23,24} Sequencing of the cloned amplification product demonstrated agreement with the published sequence.

A cDNA fragment encoding the secretory signal peptide from human tissue plasminogen activator (TPA) was obtained

by digestion of the plasmid pST₇TPA²⁵ with *Xba* I and *Bgl* II. The amplified partial apo A-I cDNA was digested with *Bgl* II/*Xba* I, and both fragments ligated into *Xba* I cut pACCMV/pLpA²⁶ to produce pACCMV apo A-I. This resulted in an in-frame fusion of sequences encoding the TPA secretory signal sequence with sequences encoding mature human apo A-I. In the resulting fusion protein, the Gln-Asp propeptide cleavage site in native human apo A-I is replaced by the Arg-Ser site from human TPA, introducing an Asp to Ser substitution at the aminoterminus of the mature apo A-I protein. In the plasmid pACCMV apo A-I, this chimeric cDNA is positioned between the human cytomegalovirus immediate-early promoter-enhancer²⁷ and the polyadenylation transcriptional termination sequences from SV40 to form a complete transcriptional unit.

The pACCMV apo A-I plasmid ($10 \mu\text{g}$) was cotransfected into 293 cells with $5 \mu\text{g}$ of pJM17,²⁸ a plasmid containing a full-length adenovirus 5 genome, by calcium phosphate coprecipitation using a glycerol shock to boost transfection efficiency. Homologous recombination between these plasmids results in the formation of a recombinant adenovirus genome of packageable size in which the CMV apo A-I fusion gene replaces the native adenovirus early region 1. The adenovirus E1A gene product, required for expression of native adenoviral genes, is supplied in *trans* from a copy of early region 1 integrated into the 293 cell genome.²⁹ Thus, in 293 cells, the recombinant viral genome is efficiently replicated and packaged into infectious viral particles.

After transfection, monolayers of 293 cells were overlaid with 0.65% noble agar in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 2% fetal bovine serum

(FBS, Hyclone). Plaques representing foci of lytic infection became visible 8 to 15 days after transfection, and agar plugs containing the plaques were picked using a sterile Pasteur pipette. Plugs were suspended in 0.5 mL DMEM, subjected to one freeze-thaw cycle, and the resulting suspension (plaque lysate) was used to infect fresh, confluent monolayers of 293 cells. Infected cells were incubated until extensive cytopathic effect was observed.

The identity of recombinant viruses was determined by restriction analysis and Southern blotting of viral DNA prepared from productively infected 293 cells. Infected monolayers were lysed in 0.6% SDS, 10 mmol/L EDTA, pH 8.0, and digested with 20 µg/mL proteinase K for 1 hour at 37°C. High-molecular-weight DNA was precipitated by the addition of 0.25 vol 5 mol/L NaCl and incubation on ice for 16 hours and pelleted by centrifugation at 12 000g for 15 minutes at 4°C. DNA was purified from the supernatant by phenol/chloroform extraction and ethanol precipitation. After digestion with appropriate restriction endonucleases, viral DNA was electrophoresed in 1% agarose gels and transferred to nylon membranes (Nytran, Schleicher and Schuell) by capillary blotting. Blots were hybridized with probes labeled with ³²P by oligonucleotide-primer synthesis from the parental plasmid DNA. Hybridized blots were imaged using a Molecular Dynamics Phosphorimager and ImageQuant software to demonstrate the presence of the appropriate insert.

The recombinant adenovirus AdRR5, which lacks an inserted gene in the E1 position, was generated from pACRR5³⁰ and pJM17 in the same manner. The plasmid pACMV TPA was constructed by ligating the *Xba* I fragment encoding human TPA from pST, TPA²⁵ into *Xba* I-digested pACMV pLPA, and the resulting plasmid was used to generate the recombinant adenovirus AdCMV TPA. Generation of AdCMV Luc, a recombinant adenovirus encoding firefly luciferase, has been described previously.³¹

Preparation of Purified Viral Stocks

Secondary stocks of the recombinant virus were produced by infection of confluent monolayers of 293 cells grown in 10-cm tissue culture dishes. Monolayers were infected by addition of primary virus stock directly to culture plates. Infected cells were incubated at 37°C until >90% the cells showed cytopathic effect, then lysed by one freeze-thaw cycle before the medium/lysate was collected.

Large-scale production of recombinant adenovirus was performed essentially as described previously^{32,33} by infecting confluent monolayers of 293 cells grown in 15-cm tissue culture plates with primary stock at a multiplicity of infection of 0.1 to 1.0. Infected monolayers were lysed with NP40 (final concentration, 0.1%) when >90% of the cells showed cytopathic changes. Virus-containing extracts were centrifuged at 12 000g for 10 minutes at 4°C to remove cellular debris. Viral particles were precipitated by the addition of 0.5 vol of 20% polyethylene glycol (PEG) 8000, 2.5 mol/L NaCl, and incubation on ice for 1 hour. Precipitated virus was collected by centrifugation at 12 000g for 20 minutes. The resulting pellet was resuspended in 20 mmol/L Tris HCl, pH 8.0, containing CsCl ($\rho=1.1$ g/mL), layered over a discontinuous ($\rho=1.3$ to 1.4 g/mL) density gradient, and centrifuged for 2 hours at 20 000 rpm in a Sorvall TH641 rotor at 4°C. Recombinant virus was harvested from the 1.3 to 1.4 interface and desalted by chromatography on Sepharose CL4B in an isotonic saline buffer (10 mmol/L Tris HCl, pH 7.4, 137 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂). Purified virus eluting in the void volume was collected and, after addition of sterile bovine serum albumin to a final concentration of 0.1 mg/mL, snap-frozen in liquid N₂ and stored at -80°C until used. The titer of infectious viral particles in purified stocks was determined by plaque assay in monolayers of 293 cells.³³ Purified viral stocks of >10¹⁰ pfu/mL were routinely obtained.

Infection of Cultured Cells

CV-1 cells were cultured in 10-cm tissue culture dishes in DMEM supplemented with 10% FBS (Hyclone) and infected by the addition of various amounts of virus diluted into 1.5 mL of serum-free media directly to the culture plates. Duplicate 10-cm plates were infected for 1 hour with 10⁷, 10⁸, and 10⁹ pfu AdCMV apo A-I (multiplicities of infection [MOI] of approximately 1, 10, and 100). As a control, duplicate plates were infected with AdCMV TPA at MOI of 10 or were mock-infected with serum-free media alone. After 1 hour of exposure to virus, the infecting media was aspirated and the cells reincubated in DMEM supplemented with 10% FBS. After 24 hours, medium was replaced with 8 mL of serum-free DMEM supplemented with 250 µg/mL penicillin and 50 µg/mL streptomycin for 10 days at 37°C. Aliquots of conditioned media were obtained at various intervals after infection for determination of apo A-I concentration.

Animal Experiments

All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. Female BALB/c mice were anesthetized by intraperitoneal injection of 200 µg of Nembutal. An internal jugular vein was exposed through a combination of sharp and blunt dissection, and 0.1 to 0.25 mL of purified recombinant adenovirus stocks (approximately 1 × 10⁹ pfu/mL) was injected intravenously using a tuberculin syringe under direct visualization. Hemostasis was obtained by direct pressure and the incision closed with wound clips. Animals were allowed to recover on a warming tray before being returned to cages, where they were provided with food and water ad libitum. Samples of tail blood were obtained from reanesthetized animals at varying intervals after infection for determination of circulating apo A-I and cholesterol levels. After 1 to 26 days, animals were killed by intraperitoneal injection of 2 mg of Nembutal and exsanguinated. In addition, livers were harvested from some animals for isolation of nucleic acids or histological analysis.

Immunoprecipitation of Apo A-I

Human apo A-I was immunoprecipitated from medium conditioned by AdCMV apo A-I-infected and control CV-1 cells and from serum obtained from control (AdRR5 infected) and AdCMV apo A-I-infected mice using a commercially available goat anti-human apo A-I antibody (Sigma). Precipitated protein was electrophoresed on SDS-15% polyacrylamide gels and stained with Coomassie blue.

Analysis of RNA in AdCMV Apo A-I-Infected Cells and Liver From Infected Animals

Total cellular RNA was prepared from uninfected and AdCMV apo A-I-infected CV-1 cells (48 hours after infection, MOI approximately 100) using the RNA STAT-60 reagent as directed by the supplier (Tel-Test "B"). Similarly, total RNA was prepared from homogenized liver samples obtained from AdRR5 and AdCMV apo A-I-infected mice. Purified RNA (20 µg) was size-fractionated by electrophoresis in formaldehyde/1% agarose gels, capillary-blotted to nylon membrane (Nytran, Schleicher and Schuell), and hybridized against human apo A-I sequences uniformly labeled with ³²P by oligonucleotide-primer synthesis. Hybridized blots were imaged using a Molecular Dynamics Phosphorimager and ImageQuant software.

Lipoprotein Fractionation

Pooled serum from groups of uninfected, AdRR5-infected, and AdCMV apo A-I-infected mice was brought to a density of 1.21 g/mL by the addition of solid KBr, layered over a cushion of 1.21 g/mL KBr, and centrifuged for 10 hours at 35 000 rpm and 4°C in a Sorvall TH641 rotor.^{34,35} The lipoprotein fractions were collected from the top of the density buffer and chromatographed on a Superose 6 column essen-

tially as previously described.³⁶ Absorbance of the column eluate at 280 nm was monitored continuously, and 0.5- to 1-mL fractions were collected for determination of apo A-I, total protein, cholesterol, and triglyceride concentrations.

Assay Procedures

Apo A-I

Apo A-I concentrations in conditioned medium and serum samples were determined using a commercially available immunoturbidometric assay (Sigma) with minor modifications. Aliquots (5 µL) of each sample were mixed with 50 µL of the antibody reagent (goat anti-human apo A-I) in 96-well, flat-bottom ELISA plates (Corning) and incubated for 15 minutes at room temperature. For determining lower concentrations of apo A-I (after lipoprotein flotation and chromatographic fractionation), 50 µL of sample was mixed with 50 µL of antibody reagent. The absorbance was read at 340 nm on a Molecular Devices Thermomax plate reader and analyzed using Softmax software. All apo A-I determinations were performed in duplicate. Standard curves were constructed from apo A-I standards provided by the kit supplier.

Cholesterol

Cholesterol levels in serum were determined using a commercially available cholesterol oxidase-based assay kit (Sigma) by a modification of the assay protocol suggested by the supplier. Aliquots (5 µL) of serum samples were mixed with 100 µL of the enzyme reagent in 96-well, flat-bottom microtiter plates and incubated at 37°C for 5 minutes. For determining lower concentrations of cholesterol, a 100-µL sample was mixed with 100 µL of reagent. Absorbance was read at 490 nm in the Molecular Devices plate reader and analyzed using Softmax software in comparison to standard curves generated using commercial cholesterol standards. All assays were performed in duplicate.

HDL Cholesterol

The concentration of HDL cholesterol in serum samples was determined in the same assay following selective precipitation of low- and intermediate-density lipoproteins. Aliquots (20 µL) of serum were incubated with 4 µL of phosphotungstic acid in 96-well microtiter plates for 5 minutes at room temperature and precipitated lipoproteins pelleted by centrifugation for 10 minutes at 3000 rpm in a Sorvall RT6000. The resulting supernatant was assayed for cholesterol as described.

Triglycerides

Serum triglycerides were determined using a commercially available enzymatic assay (Sigma). Aliquots (5 µL) of serum were incubated with 50 µL of fresh enzyme reagent for 10 minutes at 37°C in 96-well microtiter plates and the absorbance at 490 nm determined in the plate reader. For lower concentrations of triglyceride, 50 µL of sample was mixed with 50 µL of reagent. All assays were done in duplicate and compared with a standard curve constructed from the commercially supplied triglyceride standards.

Evaluation of Hepatotoxicity

Gamma-glutamyl transpeptidase, aspartate aminotransferase, and serum bilirubin levels were similarly determined using commercially available kits (Sigma) according to protocols provided by the supplier modified only by scaling volumes for microtiter plate assays. Absorbance in these assays was determined using a Molecular Devices microtiter plate reader and Softmax software and quantified by comparison against commercial standards.

Recombinant DNA Techniques

Manipulation of recombinant DNA was performed essentially as described.³⁷ Enzymatic reactions were performed under conditions recommended by the suppliers.

Histopathology

Sections of liver obtained from mice at various intervals after infection with AdCMV apo A-I were fixed in 0.25% glutaraldehyde in phosphate buffered saline for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for photomicrography.

Data Analysis

Serum levels of human apo A-I, serum lipid concentrations, and circulating enzyme activities were compared using ANOVA. To determine significance between groups, pairwise post hoc analysis with Scheffé's method was used.³⁸ For all determinations, significance was assumed at $P < .05$.

Results

Cells Infected With AdCMV Apo A-I Express Immunoreactive Human Apo A-I

To determine whether cells infected with AdCMV apo A-I would synthesize and secrete human apo A-I, CV-1 cells cultured in 100-mm tissue culture dishes were infected with 10^7 , 10^8 , and 10^9 pfu (corresponding multiplicities of infection of approximately 1, 10, and 100). Northern blotting of total cellular RNA isolated 48 hours after infection demonstrated expression of a single species of RNA (approximately 1.2 kb in size) hybridizing to the human apo A-I probe. RNA from uninfected cells demonstrated no hybridization (Fig 2). Time- and dose-dependent accumulation of immunoreactive human apo A-I was observed in medium conditioned by AdCMV apo A-I-infected cells but not control cells (Fig 3). Cells infected at a multiplicity of 100 secreted immunoreactive apo A-I protein at a rate of approximately $47 \mu\text{g}/10^6 \text{ cells}$ per 24 h over a 10-day period after infection.

AdCMV Apo A-I-Infected Mice Express Human Apo A-I

To determine whether infection of intact animals with AdCMV apo A-I would result in accumulation of human apo A-I protein in serum, 13 BALB/C mice were infected by intravenous injection of purified virus. High levels of human apo A-I were detected in serum from infected mice 1 day ($251 \pm 103 \text{ mg/dL}$, $n=6$) and 5 days (Table) after infection. Serum from uninfected animals or from animals infected with the irrelevant recombinant adenoviruses AdCMV Luc and AdRR5 demonstrated no immunoreactive material detectable above background ($<5 \text{ mg/dL}$), confirming that the endogenous murine protein did not significantly cross-react in the immunoturbidometric assay. Similarly high levels of human apo A-I were observed in C57B/6 mice infected with AdCMV apo A-I (data not shown).

SDS-polyacrylamide gel electrophoresis of protein immunoprecipitated from serum of infected mice and from medium conditioned by AdCMV apo A-I-infected CV-1 cells by goat anti-human apo A-I antiserum revealed a 28-kD band comigrating with the authentic human protein (Fig 4).

While high levels of the recombinant human protein were observed in mice 5 days after infection, levels



Fig 2. Northern blot analysis of RNA isolated from uninfected and AdCMV apolipoprotein (apo) A-I-infected CV-1 cells. Total cellular RNA (20 µg) was size-fractionated by electrophoresis in formaldehyde-1% agarose, transferred to nylon membrane, and probed for human apo A-I sequences with a fragment of the human apo A-I cDNA uniformly labeled with 32 P by oligonucleotide-prime synthesis.

declined to <10% of maximal by 12 days after infection and were essentially undetectable at 21 days (data not shown). Northern blotting of RNA isolated from the livers of mice 5, 12, and 26 days after infection with AdCMV apo A-I confirmed that expression of the foreign gene was extinguished at the later times (Fig 5).

Expression of Human Apo A-I Increases HDL Cholesterol

To determine whether expression of human apo A-I produced a significant alteration in circulating lipids, apo A-I, total and HDL cholesterol, and triglycerides were assayed in serum samples obtained from uninfected animals and from mice infected with 1×10^9 pfu AdCMV apo A-I or control viruses 5 days after infection (Table). Total serum cholesterol was approximately 47% greater in AdCMV apo A-I-infected mice than in control mice. HDL cholesterol levels, determined after selective precipitation of other lipoproteins with phos-

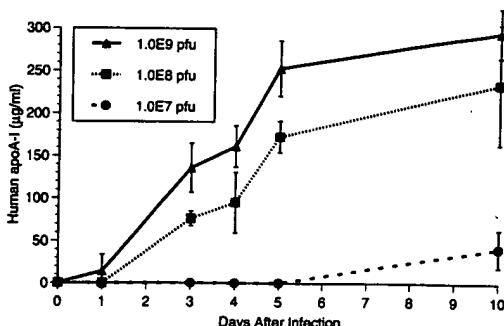


Fig 3. Accumulation of human apolipoprotein (apo) A-I in medium conditioned by CV-1 cells infected with AdCMV apo A-I. CV-1 cells were infected with AdCMV apo A-I at multiplicities of infection of approximately 1, 10, and 100. At the indicated intervals, aliquots of conditioned medium were removed and assayed for apo A-I using a commercial immunoturbidometric assay. Medium conditioned by mock-infected cells or CV-1 cells infected with the control virus AdCMV TPA at a multiplicity of 10 secreted no detectable human apo A-I in the conditioned media at any time point (data not shown).

photungstic acid, were approximately 35% greater in AdCMV apo A-I-infected animals. These results are similar to those previously observed in mice transgenic for a copy of the human apo A-I gene.¹⁸⁻²¹

To further examine alterations in serum lipoproteins induced by overexpression of apo A-I, pooled sera from uninfected mice, from animals infected with AdCMV apo A-I, and from mice infected with AdRR5 was fractionated by KBr density gradient ultracentrifugation. Lipoprotein-containing fractions (density, $\rho < 1.21$ g/mL) were further separated by chromatography on Superose 6, and fractions eluting from the column were assayed for protein, cholesterol, and human apo A-I (Fig 6). The human apoprotein coeluted with the predominant cholesterol peak, suggesting incorporation of human apo A-I into HDL particles of appropriate density and size.

Mice infected with AdCMV apo A-I also demonstrated an increase in serum triglycerides (199 ± 81 mg/dL) at 5 days compared with control infected (97 ± 41 mg/mL) or uninfected (125 ± 40 mg/mL) mice, respectively ($P \leq .05$ for both groups compared with AdCMV apo A-I-infected mice). Serum triglycerides in mice infected with either AdCMV Luc or AdRR5 averaged 97 ± 41 mg/dL, a level not significantly different from levels in uninfected animals (mean, 125 ± 40 mg/dL). This suggests that the rise in serum triglycerides is related to overexpression of human apo A-I rather than infection with adenovirus. In general, animals infected with lower doses of AdCMV apo A-I demonstrated smaller increases in serum triglycerides (data not shown). The elution pattern of triglyceride containing lipoproteins from the Superose 6 column was not qualitatively different for serum from AdCMV apo A-I-infected mice in comparison to uninfected or AdRR5 infected mice, although the levels of triglycerides eluting in the very low-density lipoprotein (VLDL) peak were increased.

Infection With AdCMV Apo A-I Is Associated With Transient Hepatic Injury

Infection of experimental animals by adenoviruses has been associated with lymphocytic infiltration of

Human Apolipoprotein A-I and Lipids in Control and Infected Mice

	Human Apo A-I, mg/dL	HDL Cholesterol, mg/dL	Total Cholesterol, mg/dL	Triglycerides, mg/dL
AdCMV apo A-I-infected mice (n=13)	168±68*†	77±14*†	135±33*†	199±81*†
Control infected mice (n=9)	2±3‡	57±7‡	92±9‡	97±41‡
Uninfected mice (n=10)	1±1	64±8	87±12	125±40

Apo indicates apolipoprotein; HDL, high-density lipoprotein. Values are expressed as mean±SD.
*P≤.05 vs uninfected mice; †P<.05 vs control infected mice; ‡P=NS vs uninfected mice.

several target tissues, including liver and lung.³² To assess whether administration of AdCMV apo A-I to normal mice produced a lymphocytic hepatitis, liver tissue was harvested from animals 5, 12, and 26 days after infection for histological examination. As anticipated from prior observations, a prominent lymphocytic infiltrate was observed in hepatic tissue 5 days after infection (data not shown). Similar infiltrates were observed in liver tissue harvested from AdRR5-infected mice, suggesting a response to the viral vector rather than to the encoded foreign gene. By 12 days, the inflammatory response appeared significantly less, and 26 days after infection, no residual infiltrate was observed. Serum γ -glutamyl transpeptidase (GGT) and

bilirubin levels were not increased in AdCMV apo A-I-infected mice 5 days after infection versus AdRR5-infected animals (GGT, 8±1.5 versus 9±0.5 U/mL, P=NS; bilirubin, 0.8±0.3 versus 0.8±0.2 mg/dL, P=NS). Assessment of serum AST levels was confounded by variable degrees of ex vivo hemolysis.

Discussion

The studies described in this report demonstrate that mice infected with a recombinant adenovirus containing a gene encoding human apo A-I efficiently synthesize and secrete the human protein into serum. Protein expressed from the foreign gene is apparently incorporated into physiological HDL particles, and infected

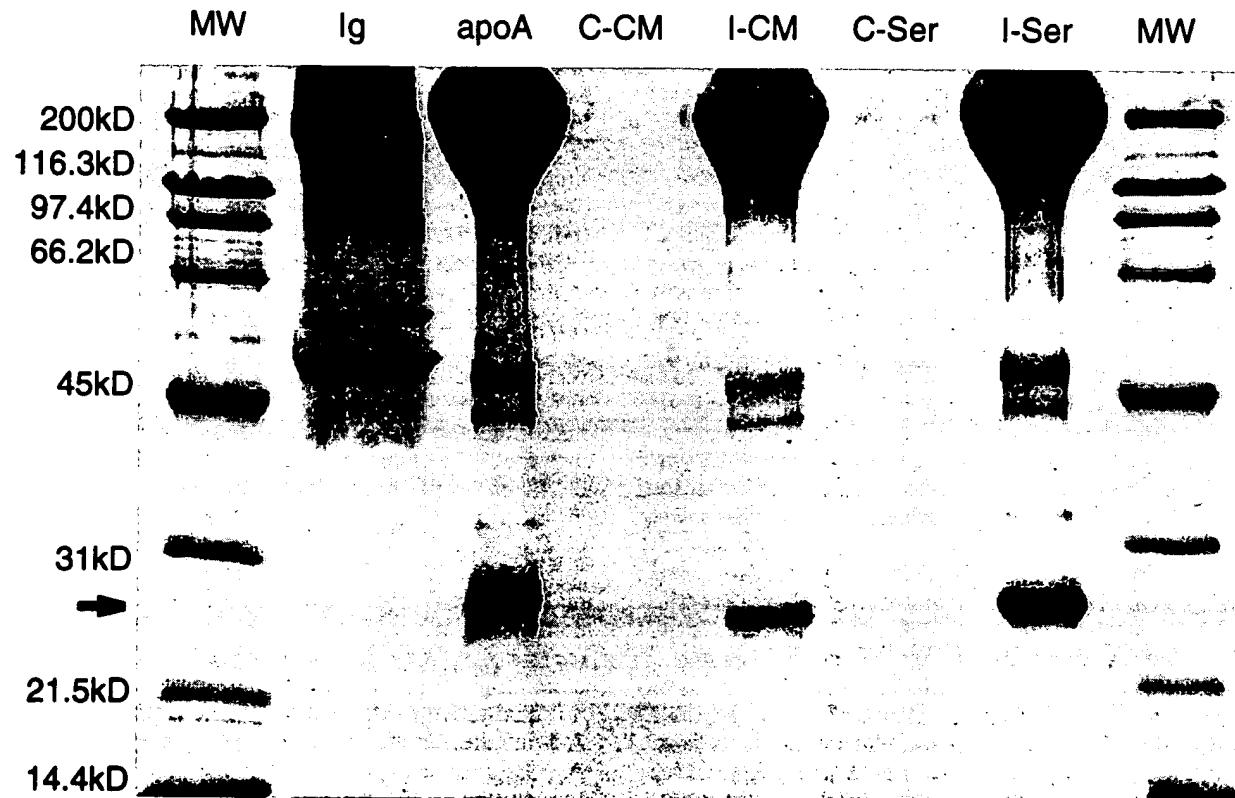


Fig 4. Immunoprecipitation of human apolipoprotein (apo) A-I. Aliquots of conditioned medium or mouse serum were incubated with goat anti-human apo A-I antiserum. Precipitated protein was collected by centrifugation, separated by electrophoresis in an SDS-15% polyacrylamide gel, and stained with Coomassie blue. Lanes: MW indicates molecular weight standards; Ig, commercial (Sigma) anti-human apo A-I antiserum; apoA, immunoprecipitated purified human apo A-I; C-CM: medium conditioned for 5 days by uninfected CV-1 cells; I-CM, medium conditioned for 5 days by CV-1 cells after infection with AdCMV apo A-I; C-Ser, serum from a mouse 5 days after infection with AdRR5; I-Ser, serum from a mouse 5 days after infection with AdCMV apo A-I. Arrow indicates the position (28 kD) at which human apo A-I migrates.

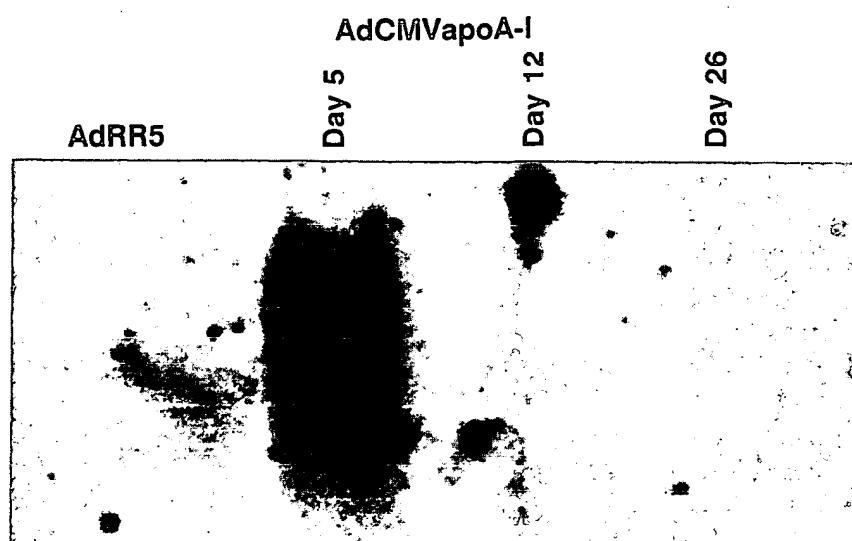


FIG 5. Northern blot of mouse liver RNA. Total cellular RNA was isolated from the livers of mice 5, 12, or 26 days after infection with AdCMV apolipoprotein (apo) A-I or 5 days after infection with AdRR5, separated by electrophoresis in formaldehyde-1% agarose gels, transferred to nylon membrane, and probed for human apo A-I sequences with a fragment of the human apo A-I cDNA uniformly labeled with ^{32}P by oligonucleotide-primed synthesis.

animals demonstrate a significant increase in circulating HDL cholesterol levels. These observations imply that the rate of apo A-I synthesis and secretion from the liver is an important determinant of circulating HDL cholesterol. The magnitude of the increase in HDL cholesterol observed after adenovirus-mediated gene transfer is similar to that observed in animals transgenic for a copy of the human apo A-I gene. The increase in HDL cholesterol observed in these animals, moreover, is large relative to that affording significant protection against the progression of atherosclerosis in both clinical and experimental studies¹⁻⁴ and to that associated with a reduction in cardiovascular risk in humans.²

Introduction of foreign genes into somatic cells in intact animals has been achieved with a variety of vectors, including recombinant retroviruses, synthetic vectors, and recombinant adenoviruses (reviewed in References 39 through 42), and in some cases expression of cellular proteins or paracrine growth factors has

been sufficiently efficient to produce demonstrable physiological effects. In vivo somatic cell gene transfer to produce physiologically meaningful quantities of a serum protein, however, has not been previously demonstrated. The experiments described here demonstrate that adenovirus-mediated gene transfer can result in significant overexpression of a serum protein, highlighting the high efficiency of gene transfer achievable with this vector. While this high efficiency makes adenovirus a very attractive vector for the introduction of foreign genetic material into somatic cells in vivo, our observations also emphasize several important limitations.

Expression of human apo A-I declined rapidly, falling to <10% of peak levels 12 days after infection. In part, this may reflect extinction of expression from the human CMV promoter used in these experiments.⁴³ Previous experiments⁴² have demonstrated that the adenovirus genome is maintained in an episomal state in quiescent somatic cells in vivo and is lost from the livers of infected animals over several weeks,⁴⁴ suggesting either that the recombinant genome is unstable in the somatic cells of adult animals or that genetically modified cells are eliminated by host immune surveillance. Prior studies of the stability of foreign gene expression after adenovirus-mediated gene transfer have yielded varying results. Stratford-Perricaudet et al⁴⁵ reported expression of β -galactosidase in skeletal muscle for up to 1 year after infection with a recombinant adenovirus. Rosenfeld et al⁴⁶ have observed expression of the CFTR gene in pulmonary epithelium for up to 6 weeks after infection, while Herz and Gerard³² described a rapid decrease in expression of a human low-density lipoprotein LDL receptor gene after intravenous administration of a recombinant adenovirus to mice. While in part these disparities may reflect the end points used to assess expression, they suggest that the stability of foreign gene expression may vary with target tissue, host, or genetic construct. For some potential applications, transient expression of a foreign gene may be sufficient to achieve specific physiological goals. Our observations suggest, however, that strategies to stabilize foreign gene expression in quiescent somatic cells of adult animals will be critical to long-term efficacy after adenovirus-mediated gene transfer.

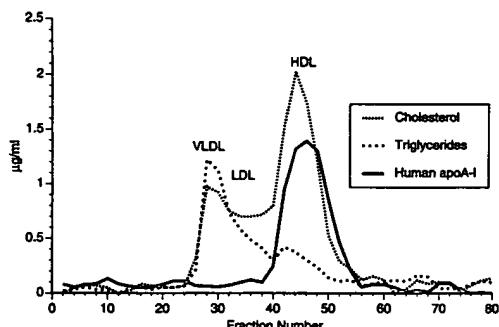


FIG 6. Analysis of density-purified lipoproteins in pooled serum from AdCMV apolipoprotein (apo) A-I-infected mice. Samples of serum obtained from mice 5 days after infection with AdCMV apo A-I were pooled and the lipoprotein containing fraction ($\rho < 1.21$) isolated by KBr density gradient ultracentrifugation. Lipoproteins were further fractionated by chromatography on Superose 6 and fractions eluting from the column analyzed for protein, human apo A-I, cholesterol, and triglycerides. Human apo A-I coeluted with the major cholesterol peak in a position corresponding to high-density lipoprotein (HDL). Cholesterol and triglyceride elution profiles for lipoprotein samples prepared from serum from uninfected and AdRR5-infected mice were qualitatively similar (data not shown). VLDL indicates very low-density lipoprotein; LDL, low-density lipoprotein.

In mice infected with AdCMV apo A-I, we observed an increase in total cholesterol of a magnitude greater than that accounted for by the observed increase in HDL cholesterol. In addition, mice infected with AdCMV apo A-I but not with AdRR5 of AdCMV Luc demonstrated an unanticipated and significant increase in serum triglyceride levels. On Superose 6 chromatography, the increased triglycerides eluted in the VLDL fraction, suggesting an indirect and previously undescribed effect of overexpression of human apo A-I on VLDL metabolism. These observations contrast with those in mice transgenic for a copy of the human apo A-I gene, in which no significant increase in serum triglycerides were observed.^{18,19} Identification of the mechanisms responsible for these alterations in lipoprotein profiles will require an analysis of endogenous murine apolipoproteins and lipoprotein turnover studies. Such might provide insight into unrecognized mechanisms regulating lipoprotein metabolism.

We observed a transient lymphocytic infiltrate in the livers of experimental animals infected with high titers of recombinant adenovirus. Similar inflammatory responses were observed in animals infected with AdCMV apo A-I and control viruses, suggesting that this response is related to the vector rather than the inserted foreign gene. Despite the histopathological abnormalities, serum γ -glutamyl transpeptidase and bilirubin levels were not increased in either AdCMV apo A-I- or AdRR5-infected mice in comparison to uninfected control animals. A similar lymphocytic hepatitis has been observed in mice infected with a recombinant adenovirus encoding the human LDL receptor,³² and inhalation of human adenovirus 5 has been reported to produce a transient lymphocytic interstitial pneumonitis in rodents in the absence of evidence of viral replication.⁴⁷ Whether this immune response results entirely from the administered load of viral antigen or reflects low-level expression of endogenous adenovirus genes by infected cells has not been clearly determined but has important implications for efforts to extend the duration of foreign gene expression after adenovirus-mediated gene transfer and potential implications for the safety of recombinant adenovirus vectors for human gene therapy.^{48,49} While to date, adverse effects on the host have been limited to transient inflammatory infiltration of target organs, detailed toxicology studies are unavailable. Moreover, it remains uncertain whether the host immune response to recombinant adenovirus contributes to the extinction of foreign gene expression or whether it will substantially inhibit repeated infection.

Despite the limitations of the current study, these experiments demonstrate the potential for somatic cell gene transfer to augment circulating apolipoprotein levels and specifically to increase circulating HDL levels. While the alterations in lipoprotein levels are transient, the duration of expression should be sufficient for physiological studies of lipoprotein metabolism, alleviating for some purposes the need to generate transgenic animals. This approach may be particularly useful in studies of altered lipoprotein metabolism in (1) larger animals more amenable to studies of vascular biology, (2) species with endogenous lipoprotein profiles more closely resembling those in humans, and (3) species in which generation of transgenic animals is difficult or impractical.

In addition, the potent antiatherogenic effects of HDL make it an attractive target for therapeutic intervention to prevent or retard the progression of atherosclerosis. Both clinical and experimental studies suggest that even minor alterations in HDL cholesterol can exert an important antiatherogenic effect, and the degree of augmentation of HDL cholesterol observed in these experiments has been associated with a protective effect in both experimental animals and humans. Further experiments to determine whether adenovirus-mediated transfer of a gene encoding apo A-I can convey protective effects against the development of vascular disease in well-characterized animal models are warranted, with the goal of evaluating the potential of a gene-based strategy to reduce cardiovascular risk.

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